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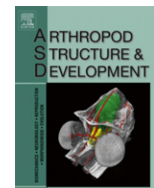

Brooding and embryonic development in the crustacean *Paragnathia formica* (Hesse, 1864) (Peracarida: Isopoda: Gnathiidae)

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ABSTRACT

The crustacean family Gnathiidae Leach, 1814 (Peracarida: Isopoda) comprises 12 genera known mostly from marine environments. Juvenile gnathiid isopods are fish ectoparasites, feeding on blood and tissue fluids in order to complete their life cycles. Gnathiid juvenile development generally includes three moults, the last involving metamorphosis to non-feeding, adult stages. The blood meal ingested by juveniles provides resources for adult survival, reproduction and embryological development. Reproductive biology in the brackish water gnathiid, *Paragnathia formica* (Hesse, 1864), is unusual amongst crustaceans, since brooding females have paired internal uterine sacs, rather than an external brood pouch. Known embryological development for *P. formica* includes three post gastrulation stages. In the current study, brooding and embryological development in this gnathiid were reexamined using histological and fluorescence methods, and by scanning electron microscopy. Novel observations were made of the blastodisc and germ cell migration within developing eggs, release of Stage 2 embryos by rupture of embryonic membranes, the *in utero* moult of Stage 2 to Stage 3 embryos, and the asynchronous development of the brood within the paired uterine sacs. These findings highlight the remarkable nature of brooding in *P. formica* and expand the paucity of knowledge of embryological development in gnathiids in general.

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1. Introduction

Crustaceans are generally oviparous, releasing eggs directly into the water column or carrying laid eggs on the carapace, or appendages, until they hatch (Barnes and Harrison, 1992). However, members of the superorder Peracarida are ovoviparous, and eggs are usually transferred from the ovaries to an external brood pouch or marsupium, typically enclosed by ventral oöstegite plates (Johnson et al., 2001). Most isopods follow this brooding pattern, though internal and semi-internal brood pouches have been reported (Harrison, 1984; Upton, 1987; Johnson et al., 2001). The brooding mechanism of the gnathiid isopod *Paragnathia formica* (Hesse, 1864) (Crustacea; Peracarida: Isopoda), is highly unusual within the subphylum Crustacea, it also differs considerably from that reported in other isopods of the family Gnathiidae Leach, 1814, and is the subject of the current study.

Gnathiids are mainly marine, but also brackish water isopods that demonstrate protelian parasitism, with juveniles being temporary parasites of fishes, and adults free living (see Smit and Davies, 2004). The semelparous females release segmented juveniles known as zuphea 1 (Z1) stages, which feed on fish blood and/or tissue fluids becoming inflated praniza 1 (P1) individuals. On leaving their hosts, P1s digest their blood meal and moult, becoming Z2s. The pattern is repeated twice more until the inflated P3 stage is reached; P3s may then be recognised as presumptive males or females and, after moulting, become non feeding adults. This final moult is cited as an example of "true metamorphosis" (see Upton, 1987), since P3 juveniles and adult stages are highly polymorphic. The classification of approximately 185 species across 12 genera within the family Gnathiidae has relied on descriptions of male gnathiid anatomy (Smit and Davies, 2004; Tanaka, 2005; Hadfield and Smit, 2008). Females have been seldom described anatomically and details of their brooding patterns are scarce. However, representatives of the gnathiid genera *Gnathia* Leach, 1814, *Caecognathia* Dollfus, 1901 and *Elaphognathia* Monod, 1926 display brooding mechanisms like those of other isopods, with developing eggs enclosed in a ventral marsupium (Monod, 1926; Wägele, 1987a; Tanaka and Aoki, 2000; Smit and Basson, 2002; Smit et al., 2003; McKiernan et al., 2005; Hadfield et al., 2008; Coetzee et al., 2009). Embryological development within the marsupial pouches of these gnathiid genera has been reported, as in Tanaka and Aoki (2000) and Smit et al. (2003), though not in great detail.

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The genus *Paragnathia* Omer Cooper & Omer Cooper, 1916 is monotypic, and *P. formica* occurs in brackish river estuaries from the United Kingdom, through Europe, to North Africa (Monod, 1926; Upton, 1987; Cadée et al., 2001; Silva et al., 2006). The extraordinary life cycle of *P. formica* is understood only generally (Stoll, 1962; Amanieu, 1963; Upton, 1987); it inhabits galleries in mud microcliffs, with each gallery containing a harem of a single male, several females and juveniles. Fertilisation has not been observed in this gnathiid, although the process occurs post-metamorphosis in other gnathiid species (Smit et al., 2003; Coetzee et al., 2009). *P. formica* displays uniqueness in its brooding mechanism among gnathiids, since the eggs are not transferred to a marsupium, but remain within the ovaries for the duration of embryological development (Monod, 1926). These internal brooding pouches were designated "uterine sacs" by Monod (1926) who first recognised the apparent individuality of this crustacean, which releases between 12 and 136 (mean brood size of 82.4) fully developed Z1 stages through vestigial oöstegite plates under the cephalosome of the female (Tinsley and Reilly, 2002).

Monod (1926) also described three post gastrulation embryonic stages occurring within the uterine sacs of *P. formica* females and subsequently Upton (1987) documented a series of female development phases, based on Monod's work. However, the brief description of developing eggs and particularly the second embryonic stage prompted our reexamination of the embryological development of *P. formica* as part of an ongoing study of this gnathiid (Manship et al., 2008). Novel observations included the appearance of the blastodisc and germ cell migration within developing eggs, the release of Stage 2 embryos by rupture of embryonic membranes, the *in utero* moult of Stage 2 to Stage 3 embryos, and the asynchronous maturation of the brood within the paired uterine sacs.

2. Materials and methods

2.1. Specimens and their developmental stages

P. formica was sampled at low tide from north facing microcliffs in salt marshes of the Dovey Estuary at Ynyshir, Ceredigion, Wales (Ordnance Survey grid reference, SN 674 973) (see Manship et al., 2008). The gnathiid was extracted from mud cores (Manship et al., 2008) and individuals sorted initially by stage. Female P3s were identified by their developing ovaries (Upton, 1987); these P3 stages, mature females and males were then maintained separately in an incubator at 16 °C with a 12 h/12 h light/dark cycle (Tinsley and Reilly, 2002) in sea water, or on moist paper in 24 well culture plates (Nunc), mimicking conditions found in the mud galleries. Exceptionally, stages were incubated together in sea water to observe their behaviour, although ripe adult females tended to shed their Z1 stages readily under these conditions and were therefore more commonly kept on damp paper, which delayed hatching.

2.2. Staging of females and nuclear staining of eggs/embryos

Uterine sac colouration (light yellow, containing early embryonic stages; orange, with early eyed embryos; dark grey, with final embryonic stages, prior to hatching) was used to stage live gravid adult females. To confirm the developmental stages within these females, eggs/embryos were removed from some ($n = 15$ females) and fixed directly in 70% ethanol, or alternatively, incubated for nuclear staining (Extavour, 2005) in phosphate buffered saline (PBS) containing 3.6 μM 4′,6 diamidino-2-phenylindole (DAPI) (Sigma) for 40 min at room temperature, rinsed in PBS, and mounted in Vectashield (Vecta Laboratories). For comparison, images of both ethanol fixed and nuclear stained eggs/embryos were captured with a Zeiss Axioskop photomicroscope with a fluorescence microscopy (FM) facility (Carl Zeiss), Nikon DN100 camera and Nikon Eclipse Net image analysis package (Nikon).

2.3. Histology

Live female P3s ($n = 20$) and staged adult females ($n = 20$) were rinsed in distilled water, and then fixed directly in standard 10% neutral buffered formalin (NBF), rinsed in PBS, and incubated in decalcifying solution for 18–24 h at 4 °C (Charmantier et al., 1987). They were then dehydrated in a graded ethanol series and paraffin wax embedded, following standard protocols (Bancroft and Gamble, 2002). Serial sections (6–8 μm) were collected on 0.01% (w/v) poly-L-lysine coated slides and stained with standard haematoxylin and eosin (H & E) (Bancroft and Gamble, 2002). Images of sections were then captured by light microscopy (LM) with a Nikon Eclipse 80i photomicroscope, Nikon DS-5M camera and Nikon NIS 2.10 image analysis system (Nikon).

2.4. Scanning electron microscopy (SEM)

Live P3 juveniles ($n = 4$), staged adult females ($n = 40$), and isolated eggs/embryos were fixed rapidly in ice cold 70% ethanol, rehydrated in distilled water and then post fixed according to Felgenhauer (1987). They were then dehydrated in a graded ethanol series, incubated in 1:1 absolute ethanol to hexamethyldisilazane (HMDS) (Sigma) for 15 min and dried overnight in 100% HMDS which was allowed to evaporate. Following drying, whole female P3s and adult females were placed ventral side down on double sided adhesive carbon tabs attached to metal stubs; the dorsal uterine sacs of adult females were then dissected to reveal their contents. Groups of isolated eggs and embryos were arranged without orientation on similar carbon tabs. All specimens (P3s, adult females and eggs/embryos) were then sputter coated with gold (Polaron E5075) and examined with a Zeiss Evo 50 scanning electron microscope (Carl Zeiss). Electron micrographs were taken under variable or high pressure scanning conditions.

3. Results

3.1. Egg development in praniza 3 females

Eggs developed within inflated P3 females (Fig. 1A) from two ovarian strips stretching dorsally between pereonites (peraeon segments) three and seven, the vitellin content of the eggs giving the live premoulting P3s a characteristic yellow colouration. Histological sections through this stage revealed closely packed eggs in two broad bands, or uterine sacs (Monod, 1926), developing around the anterior hindgut remnant (Fig. 1B) and dorsal to the paired digestive glands. Eggs occupied most of the dorsal peraeon and the premoult status of the P3 females was evident from a layer of dark cells beneath the juvenile cuticle (Fig. 1B, C). Isolated female P3s rarely survived the moult to adult females in the laboratory. Furthermore, in the presence of males, neither pairing/fertilisation of late P3s, nor of newly moulted adult females (below), were evident under laboratory conditions.

3.2. Early egg/embryonic development and morphogenesis in mature females

Eggs (approximately 200–300 μm in diameter), possessed abundant vitellin and lipid droplets and gave the uterine sacs of newly moulted, live females a yellow appearance, as in the female P3 stages. In SEM and histological preparations of such females, the eggs lay within two uterine sacs occupying most of the thoracic

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